Receptor Activator of Nuclear Factor-Kappa B Ligand, Osteoprotegerin and Interleukin-17 Levels in GCF of Chronic, Aggressive Periodontitis and Type 2 Diabetes

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Abstract: Background: The aim of this study is to compare the levels of osteoclastogenesis-related factors sRANKL and OPG and their ratios as well as the level of IL-17 in gingival crevicular fluid (GCF) from subjects with chronic periodontitis, generalized aggressive periodontitis and controlled type 2 DM patients with chronic periodontitis. Methods: GCF samples and clinical periodontal parameters were randomly obtained from sixteen patients with chronic periodontitis, 16 patients with aggressive periodontitis, 16 patients with controlled diabetes type II and 12 healthy controls. Concentrations of sRANKL, OPG, and IL-17 in GCF were analyzed by enzyme-linked immunosorbent assay (ELISA). Results: Higher concentration levels of sRANKL, OPG, RANKL/OPG ratios and IL-17 in the three diseased (experimental) periodontitis groups compared to the control group. Aggressive periodontitis group showed higher concentration level of GCF IL-17 in GCF significantly higher than both chronic periodontitis and diabetic groups. Significant There was positive correlation of sRANKL, IL-17, and sRANKL/OPG ratio in GCF with the clinical parameters ($P < 0.01$). Conclusion: GCF total amount of sRANKL, OPG were significantly increased in periodontal disease, supporting its role in the alveolar bone changes developed in this disease. Th17 responses may be characteristic of AgP, and IL-17 may play a role in the pathogenesis of aggressive periodontitis.

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Key words: Periodontitis, Diabetes type 2, RANKL, OPG, IL-17, GCF, ELISA.

1. Introduction:

Chronic periodontitis (CP) and aggressive periodontitis (AgP) are bacteria-induced infections affecting the periodontium and resulting in the loss of tooth attachment and bone resorption. Aggressive periodontitis is characterized by a rapid and severe periodontal destruction in young systemically healthy subjects, and can be subdivided into localized and generalized forms according to the extension of the periodontal destruction.[1] Lipopolysaccharides and other virulence factors of periodontal pathogens have been shown to promote a host-mediated, tissue-destructive immune response. The nature of tissue destructin may be influenced by microbial, environmental, behavioral and genetic factors.[2] There is evidence that a local autoimmune reaction may participate in the onset and persistence of the aggressive periodontitis.[3] Systemic diseases that affect the host response, such as diabetes mellitus (DM) either type 1 or type 2 may potentiate the severity of periodontal disease and accelerate bone resorption.[4] Considerable evidence supports DM as a risk factor for periodontal diseases because the incidence, progression, and severity of periodontal diseases are higher in subjects with type 2 DM than in those without DM.[5]

The hallmark of periodontal diseases is bone resorption. This is mainly an action of the innate and adaptive immune mechanisms.[6] The mechanism of bone resorption and remodeling is coordinated by the interaction among the receptor activator of nuclear factor-kappa B (RANK), RANK ligand (RANKL), and its decoy soluble receptor osteoprotegerin (OPG). The expression of these receptors is regulated by several inflammatory mediators and bacterial products. RANK protein expression has been detected in normal dendritic cells, CD4 and CD8 T lymphocytes, osteoclast monocytic precursors, and endothelial cells. RANK remains expressed on osteoclast lineage cells throughout their life span.[7] RANKL is a homotrimeric transmembrane protein that is expressed as a membrane-bound and a secreted protein, which is derived from the membrane form as a result of either proteolytic cleavage or alternative splicing.[8] Many cell types express RANKL, including osteoblasts, periodontal ligament fibroblasts, gingival fibroblasts and endothelial cells. More than 50% of T cells and 90% of B cells express RANKL in periodontitis tissue, whereas less than 20% of either B cells or T cells showed RANKL expression in healthy gingival tissue.[9]

RANKL functions both as a membrane anchored molecule and as a soluble molecule, both forms bind to RANK. The binding of RANKL to RANK elicits osteoclastogenesis and promotes the activation and survival of osteoclast cells, which, in turn, results in bone resorption.[10]

Osteoprotegerin (OPG), also known as osteoclastogenesis inhibitory factor (OCIF), is a secreted TNF receptor member that is expressed ubiquitously by many types of cells and tissues. OPG is produced by osteoblasts,[11] endothelial cells and vascular smooth muscle cells.[12] OPG binds RANKL and thereby prevents activation of its single cognate RANK. Osteoclast activity is likely to depend, at least in part, on the relative balance of RANKL and OPG.[13] Serum OPG is significantly increased in both type 1 and type 2 diabetic patients.[14] There is growing evidence that Interleukin-17 (IL-17), produced by a subset of T-helper cells (Th17) play a role in inflammatory reaction, autoimmunity, and antimicrobial responses in a variety of infectious and inflammatory diseases at mucosal surfaces.[15] IL-17 has been found in patients with relatively severe periodontitis, where it could potentially contribute to bone destruction.[16] Also, IL-17 and RANKL were positively correlated to each other and to RAR-related orphan receptor gamma(RORγt) in inflamed tissue of periodontitis patients.[17]

There was a positive correlation between the level of gingival crevicular fluid RANKL/OPG ratio and clinical parameters; periodontal pocket depth and clinical attachment loss suggesting that elevated levels of local RANKL is responsible for periodontal bone resorption.[18]

Therefore, the aim of this study is to compare the levels of sRANKL and OPG and their ratios as well as the level of IL-17 in gingival crevicular fluid (GCF) from subjects with chronic periodontitis, generalized aggressive periodontitis and in type 2 DM patients with chronic periodontitis. Our hypothesis is that aggressive periodontitis may be associated with an autoimmune reaction and increased IL-17 expression. Also, RANKL/OPG ratio may be altered in diabetic individuals. It has been proposed that individuals with type 2 DM display certain features of inflammation and immunity that can alter the pathogenesis of periodontal diseases.

2. Materials and Methods:

Study Population:

Sixty subjects (age range: 26-70 years) were selected from those attending the outpatient clinic, Department of Oral Medicine, Periodontology and Oral Diagnosis, Faculty of Oral and Dental Medicine, Cairo University, Cairo, Egypt, between April 2011 and February 2012. It was a clinical randomized controlled study which included four groups: 16 patients with chronic periodontitis, 16 patients with aggressive periodontitis, 16 patients with controlled diabetes type 2 and 12 controls. Subjects were randomly assigned using a coin toss. The assignment of subjects to the groups was carried out by the clinic coordinator remote from the study. The randomization code was held centrally by the clinic coordinator and was not broken until completion of the data analysis. Detailed medical history of each subject was obtained according to the detailed questionnaire of the modified Cornell Medical Index.[19]

Inclusion Criteria:

Patients were diagnosed with moderate-to-advanced generalized chronic periodontitis, or generalized aggressive periodontitis based on the clinical and radiographic criteria proposed by the 1999 World Workshop for Classification of Periodontal Diseases and Conditions. [20] The criteria for entry were a minimum of 14 natural teeth, excluding third molars, with at least five to six teeth had sites with probing depth =6 mm and attachment loss =5 mm and radiographically determined bone loss.

Data concerning the duration and medication were obtained from diabetic patient's medical record. All subjects had presented with type 2 DM diagnosis for =5 years and under a controlled therapy.

A blood sample from each subject was taken to measure glycated hemoglobin (HbA1c). Blood samples were collected in a single laboratory and expressed as, a percentage with a normal healthy range of 4.5% to 8%. Therefore, subjects who had HbA1c values =8% were assigned to be well-controlled. Patients who presented HbA1c levels >8% were assigned to be poorly controlled and were excluded from the study.

Exclusion Criteria

Exclusion criteria were: pregnancy, lactation, current smoking or even within the last 5 years. All patients did not have any systemic illness that could affect the progression of periodontal disease and had not received any periodontal therapy and/or antibiotic, non-steroidal anti-inflammatory therapies during the previous 6 months prior to the study. Subjects with periapical pathology, orthodontic appliances, and multiple systemic complications of DM were also excluded from the study.

Twelve patients were selected from periodontally healthy volunteers who either patients were presenting for other dental treatment or University staff members. The study protocol was approved by the Ethic Committee of Faculty of Oral

and Dental Medicine. And it was explained to all participants, and informed consent forms were signed.

Clinical Monitoring

Clinical examination was performed by one calibrated examiner. For each patient, individual number of teeth presents and the clinical parameters were documented, excluding the third molar. The following periodontal parameters were evaluated on study sites: plaque index (PI),[21] gingival index (GI),[22] probing depth (PD), clinical attachment loss (CAL) using William's graduated periodontal probe to the nearest 0.5 mm.

Collection of Gingival Crevicular Fluid (GCF)

GCF was sampled one week after clinical examination so as not to alter its nature and to decide the most affected sites to collect GCF. After isolating the tooth with a cotton roll, supragingival plaque was removed with a curette without touching the gingival margin and dried gently with an air syringe. GCF was collected using standard filter paper strips (PerioPaper, Oraflow, Smithtown, NY) which were placed into the deepest sulci/pocket until mild resistance was felt and left in place for 30 seconds. Strips visually contaminated by blood were excluded. After GCF collection, the strips were immediately placed in Eppendorf vials containing 250 µl of phosphate buffered saline (PBS). The samples were left at 4◦C for 2 hrs and, then, they were frozen at −70◦C and stored until cytokine analysis by ELISA.

Quantification of Cytokine sRANKL, OPG and IL-17

Results were reported as concentrations of related factors in each GCF sample (as picograms per microliter) (pg/µl) per site in 30 seconds.

Aliquots of each GCF sample were assayed by ELISA to determine the level of sRANK-L, OPG, and IL-17 according to the manufacturer's recommendations. GCF was eluted from each filter paper strip into PBS as follows: each strip was lifted to the surface of the eluent, and another 350 µL of PBS was added to the strip (600 µL final volume). Samples were, refrigerated at 4◦C for another 20min and centrifuged at 10,000 rpm for 10 min. Finally, the strips were discarded. Commercial ELISA kits for sRANKL (Koma Biotech Inc., biotechnology) (Catalog No. K0331187) OPG and IL-17, (R and D systems, Quantikine, Minneapolis, MN, USA) (Catalog Number: DY805 for OPG and Catalog Number D1700 for IL-17) were used to analyze sRANKL, OPG, &IL-17. The kit employs a quantitative "sandwich" enzyme immunoassay technique. Briefly, antihuman monoclonal antibody specific for sRANKL, OPG, and IL-17 was precoated onto a 96-well microplate. Any sRANKL, OPG, and IL-17 present was bound by the immobilised antibody. After washing of unbound proteins, an enzyme-linked Horseradish Peroxidase (HRP) polyclonal antibody specific for sRANKL, OPG, and IL-17 was added to each microplate well and incubated. Then, a tetramethylbenzidine (TMB) substrate solution was added to each well. Any color developed was proportional to the amount of sRANKL, OPG, and IL-17 respectively. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution. The intensity of the color (optical density) was measured by spectrophotometer at a wavelength of 450nm (wavelength correction set to 540 nm) within 30min. A standard curve was prepared against their optical density and the concentration sRANKL, OPG, and IL-17 were determined. Sites with sRANKL, OPG or IL-17 levels below the detection limit of assay were scored as negative. The minimum detectable dose limit of human RANKL is typically less than 66 pg/µl, anti-human OPG is 36 pg/µl and the minimum detectable doses for IL-17 were less than 15 pg/µl.

Statistical Analysis

Statistical analyses were performed using SPSS 20 (Statistical Package for Scientific Studies) software program. Data were first examined for normality by the Kolmogorov-Smirnov test, and data that did not achieve normality were analyzed using non-parametric methods.

The study unit for sRANKL, OPG and IL-17 levels was the site rather than the subject because periodontitis is a site-specific disease. The primary variables were differences in the levels of sRANKL, OPG and IL-17 and the RANKL/OPG ratio. The secondary variables were clinical parameters, and plasma levels of HbA1c. The percentage of sites with PI, GI, the mean PD, CAL, and the levels of HbA1c were computed for each subject. One way ANOVA was used to asses significant of age, CAL, and IL-17 between groups. When there were significant differences by the ANOVA test, a pairwise comparison was performed by the Tukey test. Kruskal-Wallis test used to assess significance of PI, GI, PD, sRANKL, OPG, and sRANKL/OPG ratio between groups. When there were significant differences by Kruskal-Wallis test, a pair wise comparison was performed by Mann-Whitney with Bonferroni correction. Chi square test was used to detect the difference in frequencies of gender between groups. The possible correlation between the levels of sRANKL, OPG, IL-17, and sRANKL/OPG ratio and clinical parameters of sampled sites and

HbA1c% were tested by the Spearman rank correlation. The significance level established for all analyses was 5%. Except for pair wise comparison was performed by Mann-Whitney p value was adjusted by Bonferroni correction to 0.008.

3. Results

There were no subject and site dropouts during the course of the study period. Thus, a total of 60 subjects completed the study and a total of 180 GCF samples were analyzed.

Table 1: Demographic characteristic and Clinical parameters of study population

Characteristics	Chronic (n=16)	Aggressive (n=16)	Diabetic patients (n=16)	Control (n=12)
Age(years)				
- Mean ±Sd	48.37 ±6.88	33.68 ± 4.6[a]	56.56 ±7.2	33.08 ±5.68[a]
- Range	38 to 60	26 to 42	45 to 70	26 to 42
Gender (n)				
- Male	7	6	7	5
- Female	9	10	9	7
Controlled treatment regimen (n)			16	
Clinical parameters per site in 4 groups and glycemic parameters (HbA1c value) in treated diabetic type II patients				
PI median (Iq range)	2(1)a	2 (1)a	1 (0)b	1(1)b
GI median (Iq range)	2 (0.75)ab	2 (0)a	1 (1)b	1 (0.75)
PD (mm) mean ±Sd	5.06 ±0.57	6.8 ±1.22	3.75 ±1	1.42 ±0.52
CAL (mm) mean±Sd	6.31 ±1.19	8.69 ±1.92	4.06 ±1.06	0.5 ±0.52
HbA1c % mean ±Sd			7.13 ± 0.62	

Similar superscript, lowercase letters indicate statistically non significant differences by analysis of variance and Tukey test (P <0.05) within each experimental group

There was no statistically significant differences between aggressive periodontitis and control groups as regards age and gender (P >0.05).

No statistically significant differences in plaque index between chronic and aggressive periodontitis, also, between controlled type II DM and controls. As regards the gingival index, statistically significant differences were recorded between aggressive periodontitis group and type II diabetic group and between control group and other groups (P <0.05). Pd and CAL showed significant difference between all studied groups.

sRANKL, OPG and IL-17 Levels:

The concentrations (picograms per microliter) (pg/µl) of sRANKL, OPG and IL-17 levels (mean ±Sd) in the GCF per site of the 4 groups are presented in Figures 1, 2 and 3 respectively.

There was statistically significant higher level of sRANKL, OPG and IL17 in the three diseased (experimental) groups compared to control group. Diabetic group showed higher concentration of sRANKL 525 ±49.67 than both CP 437.5 ±68.26[a] and AgP groups 380 ±83.7[a] with no statistically significant differences between the mean recorded of chronic and aggressive periodontitis groups (P >0.05). Diabetic group showed statistically significant higher level of sRANKL than non-diabetic groups (P <0.05).

In the mean time OPG level in the diabetic group 425.63 ±25.55[b] was statistically significant higher than in AgP group 328 ±38.96[a] (P <0.05).

The level of IL-17 was extremely low in the GCF of control group 35 ±18.83. Aggressive periodontitis group showed higher level of GCF IL-17 significantly higher 179.38 ±52.34 than that in both CP 114.38 ±58.65[a] and diabetic groups 128.75 ±44.1[a] (P <0.05) where there was no statistically significant difference between chronic periodontitis group and type II diabetic group (P >0.05).

Figure 4 presents the GCF sRANKL/OPG ratios in the 4 groups per site. There was no statistically significant difference in the mean sRANKL/OPG ratios between the three experimental groups (AgP 1.17±0.32[a], CP 1.18±0.26[a], and DM 1.24±0.16[a] (P >0.05), but was significantly higher than the control group 0.52± 0.11

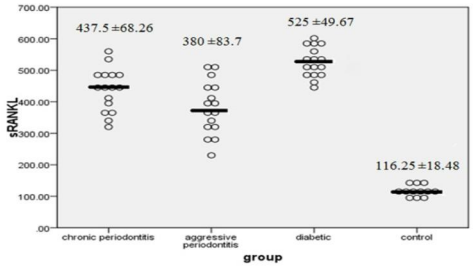

Figure 1: Distribution of concentration (pg/µl) of sRANKL in the GCF per site in the 4 groups

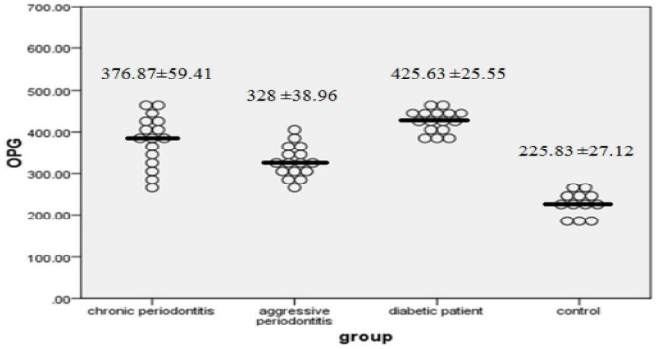

Figure 2: Distribution of concentration (pg/µl) of OPG in the GCF per site in the 4 groups

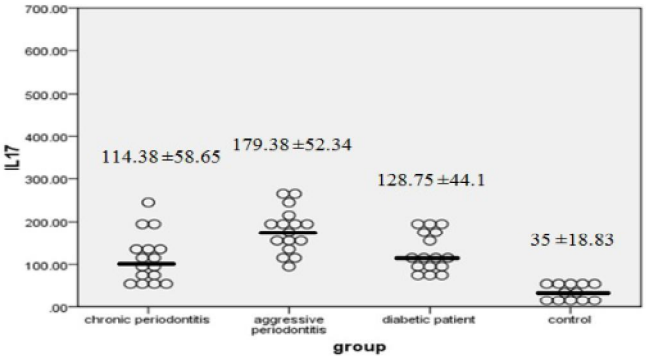

Figure 3: Distribution of concentration (pg/µl) of IL-17 in the GCF per site in the 4 groups

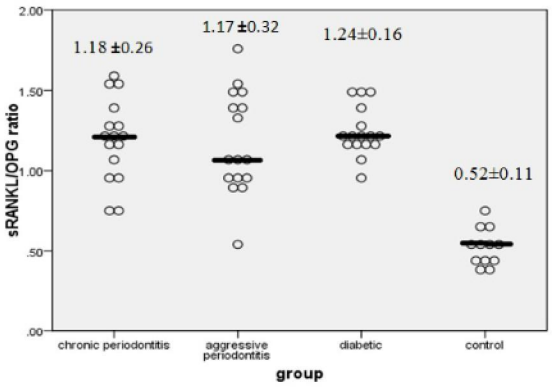

Figure 4: The ratios of RANKL/OPG in the 4 groups in GCF per site.

Correlations

Table 2 presents the correlation coefficients for total amounts and concentrations of osteoclastogenesis-related factors and clinical parameters of the sampled sites and HbA1c. The present study demonstrates significant positive correlation of sRANKL, IL-17, and sRANKL/OPG ratio in GCF with PI, GI, PD, and CAL (P <0.01).

Table 2: Correlation coefficients for the osteoclastogenesis-related factors and the clinical parameters per site and HbA1c levels

Clinical and glycemic parameter	Concentration			Ratio **sRANKL/OPG**
	sRANKL	**OPG**	**IL-17**	
PI	0.321*	0.15	0.343*	0.479*
GI	0.375*	0.192	0.373*	0.515*
PD	0.285*	0.182	0.672*	0.506*
CAL	0.286*	0.191	0.602*	0.499*
HbA1c	0.709*	-0.232	-0.18	0.609*

Significant at P <0.05 by the Spearman rank correlation test- * Significant

4. Discussion

This study compared the levels of bone-related factors (sRANKL and OPG) and cytokine (IL-17) in the gingival crevicular fluid (GCF) of systemically healthy, chronic periodontitis, aggressive periodontitis and controlled Type 2 DM patients with chronic periodontitis. We hypothesized that these three groups could exhibit different dysregulations of bone-related factors and proinflammatory cytokine profile which could affect their susceptibility to periodontal breakdown. It is important to understand the immunoinflamatory mechanisms that determine the higher susceptibility to periodontitis in patients with diabetes, compared to individuals without diabetes, as only minor periodontal microbiologic differences have been found between patients with and without diabetes.[23]

The results of the present study showed that the concentrations of sRANKL, OPG were elevated in the GCF of CP, AgP and were significantly higher in periodontitis lesions of type II diabetic patients in comparison to non diabetic groups.

These results run parallel to that reported by Ribeiro *et al.,* 2011[24] who found that Type 2 diabetes mellitus, as a whole, upregulates the levels of OPG, sRANKL and IL-17 in sites with CP. It was thought that controlling the glycemic level in type II DM patients modulate the levels of biomarkers sRANKL, OPG.[25] They found that total amounts and concentrations of sRANKL and RANKL/OPG ratios were higher in GCF of poorly controlled subjects than in well-controlled subjects. The interaction between AGE and its receptor (RAGE), which is present in different types of cells, enhances the expression of proinflammatory cytokines including interleukin (IL)-1 and -17 and tumor necrosis factor-alpha.[26]

Earlier studies reported that OPG protein was significantly lower (P < 0.05) in the periodontitis tissues [27] and in GCF of patients with periodontitis.[28] In the present study OPG concentration was elevated in all periodontitis groups. This is in accordance with the findings that found that OPG expression from gingival tissue was higher in chronic periodontitis than in healthy patients.[29] This could be explained by the observation that human periodontal ligament cells stimulated with lipopolysaccharide could inhibit osteoclastogenesis by producing higher levels of OPG than RANKL via the induction of IL-1β and TNF-α.[30] Porphyromonas gingivalis upregulated the expression of OPG by human gingival fibroblasts and in human microvascular endothelial cells via a nuclear factor-Kappa B dependent pathway; thus these cells may act as a source of OPG and thereby may play a role in regulating bone metabolism in periodontitis.[31,32] So, the regulation of the balance between bone breakdown and reformation is modulated, to a large extent, by the secreted soluble receptor OPG.

In the present study there was no statistically significant difference in sRANKL/OPG ratio among the three studied groups but were significantly higher than the control group (P<0.01) (Fig.4). Periodontal ligament fibroblasts are a source of RANKL, but concurrently produce OPG, in response to IL-1 stimulation. Periodontal ligament fibroblasts and osteoblasts express RANKL and OPG mRNA via PKA and PKC signalling, respectively.[33] Furthermore, there was increased in OPG production by human gingival fibroblasts when stimulated by lipopolysaccharide (LPS) from Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis.[32]

In this study, IL-17 was present at significantly higher concentrations in GCF from those with GAgP (179± 52.34pg/μL) than CP and diabetic patients with CP. The presence of IL-17 has been documented in sera of GAgP patients in significantly higher level than CP patients and healthy controls.[34] This may be explained in part by the autoimmune reaction in AgP.

High levels of autoantibodies directed to extracellular matrix components (type I collagen, fibronectin and laminin) were detected in the sera of AgP patients.[3] Autoantibody binding to native and ROS -modified type I and type III collagens and citrullinated peptides CCP) were observed exclusively in the sera of patients with AgP and not in those with chronic periodontitis or gingivitis.[35] Citrulline is a non-standard amino acid generated by post-translational modification of arginine residues by peptidylarginine deiminase (PAD). P. gingivalis, one of putative pathogens in AgP is the only bacterium known to express a PAD enzyme.[36]

Although the role of IL-17 in the pathogenesis of periodontal disease is poorly understood, the presence of IL-17 has been documented in GCF of periodontitis patients [37] and in periodontal tissues biopsied during periodontal surgery.[10] Elevated levels of IL-17 and bone resorptive factors RANKL, IL-1β, and IL-6 (messenger RNA and protein) as well as the presence of Th17 cells has been found in periodontal tissues from patients with periodontitis.[17,38] This suggests that IL-17 may be the link between the adaptive immune system and the innate immune system to amplify inflammation and mobilize neutrophils.

Other studies suggested dual role of IL 17, thus, in a sterile inflammatory state such as rheumatoid arthritis or other autoimmune diseases, IL-17 signalling contributes to tissue damage, whereas in bacterial infections such as periodontal diseases, IL-17 may be critical for recruiting neutrophils and/or other immune cells required to limit the spread of infection. However, IL-17RA exerts a profound bone-protective effect on PD bone loss.[39,40]

On the contrary of these results, two investigators failed to detect IL-17 in GCF samples from Japanese patients with periodontitis and from Indian patients.[41,42]

Our results showed significant positive correlations between sRANKL, IL-17, and sRANKL/OPG ratio and PI, GI, PD, and CAL (P <0.01). This result is in accordance with Bostanci *et al.,* [18] who reported a positive correlation between the GCF RANKL/OPG ratio and PD in patients with periodontitis. Other two investigators failed to report significant correlations between GCF RANKL and/or OPG concentrations and clinical measurements of disease severity in terms of PD, CAL, and inflammation with regard to bleeding on probing in patients with chronic periodontitis.[28,43]

Conclusions

High levels of bone-related factors sRANKL and OPG and cytokine IL-17 were observed in the GCF of patients with CP, GAgP and in type 2diabetic patients with CP. Patients with type 2 DM display certain features of inflammation and immune reaction that can alter the pathogenesis of periodontal diseases.

The author reports no conflicts of interest related to the study.

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